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Antimicrobial Resistance and Molecular Typing of Pathogenic *Vibrio parahaemolyticus* Isolated from Seafood in Shanghai Retail Markets

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Vibrio parahaemolyticus **is one of the most seafood-borne pathogens in China, which may cause acute gastroenteritis. We aimed to investigate the antimicrobial resistance and genotypic relationship of 41 pathogenic *V. parahaemolyticus* isolates from 342 seafood samples including 248 shrimp, 86 shellfish and 18 fish. These samples were collected from retail markets in Shanghai during March in 2011 to December in 2012. After preliminary screening in Vibrio chromogenic medium, the assumed isolates were confirmed by amplification of *tlh*, *tdh*, *trh* genes through polymerase chain reaction (PCR) and 41 *tdh* positive *V. parahaemolyticus* strains were acquired. Besides, pathogenic strains O3:K6 (Vp2) and ATCC33847 (Vp1) were tested in this study as control. By antimicrobial susceptibility testingÿthese pathogenic strains were sensitive to chloramphenicol, 14.63% were resistant to amikacin, and there were varying resistance to amoxicilin-clavulanic, gentamicin, ciprofloxacin, levofloxacin. Analysis by enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) revealed that the isolates could be grouped into six patterns. In conclusion, *V. parahaemolyticus* was commonly found in retail seafood products from Chinese aquatic market, and a few parts were pathogenic and antibiotic resistant strains which may pose a potential threat to human health.**

Key words: Pathogenic *V. parahaemolyticus*, Antibiotic resistance and ERIC-PCR.

Vibrio parahaemolyticus is a Gram-negative human pathogen which is naturally present in marine environment and frequently isolated from a variety of seafood products. Consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may lead to acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea and low fever (Yang *et al*. 2008). Food-borne outbreaks caused by *V. parahaemolyticus* were reported globally, such as Japan, United States, Taiwan, China, Korea and some European countries (Bauer *et al*. 2013; Sani *et al*. 2013; Su *et al*. 2007).

Although *V. parahaemolyticus* was frequently detected in seafood, not all strains are pathogenic. Clinical isolates of *V. parahaemolyticus* most often produce the thermostable direct haemolysin (TDH), TDH related haemolysin (TRH) or both. TDH and TRH encoded by *tdh* and *trh* genes, respectively, are now recognized as major virulence factors of *V. parahaemolyticus* (Honda *et al*. 1993; Nishibuchi *et al*. 1995). Pendru Raghunath (Raghunath *et al*. 2008) have found that seafood with total

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V. parahaemolyticus count less than hazardous limit are also not completely safe, and total *V. parahaemolyticus* count is not a reliable indicator for pathogenic *V. parahaemolyticus*. Considering the prevalence of the variants of pathogenic *V. parahaemolyticus* worldwide (García *et al*. 2009; Koralage *et al*. 2012; Vongxay *et al*. 2008), the pathogenic *V. parahaemolyticus* should be targeted for control of seafood-related transmission to humans.

Increasingly, there have been amounts of reports about antibiotic resistance in *Vibrio* species and it has been observed to be dynamic and varied with the environment (Sudha *et al*. 2012). Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment, increasing the fatality rate (Jun *et al*. 2012).

Molecular typing was shown as a useful tool for tracking the source of infection and detection of virulent strains, as well as for determining the geographical and host distribution of possible variants (Olive *et al*. 1999). ERIC-PCR is a faster and more cost-effective molecular genotyping method than pulsed-field gel electrophoresis (PFGE) or multilocus sequencing for generating information about the genetic similarity of bacterial strains (Ben-Hamouda *et al*. 2003; De la Puente-Redondo *et al*. 2000). And it was confirmed to be useful for the evaluation of genetic and epidemiological relationships among V. parahaemolyticus strains (Chena *et al*. 2012; Marshall *et al*. 1999).

Pathogenic *V. parahaemolyticus* were isolated from seafood in different markets and subjected to antibiotic resistance test to ascertain the risk posed by this microbiology. By molecular characterization, we can know the relationship among these pathogenic strains on antibiotic resistance and isolation sources. This information will be helpful to local consumers regarding the safety of various aquatic products consumed in this region, to provide data for further risk assessment and will ultimately help the seafood industry to strive for greater food safety.

MATERIALS AND METHODS

Isolation of strains

A total of 342 samples including shrimp, fish and shellfish were collected from some major aquatic markets such as Shanghai Oriental International Fisheries Market, Shanghai Tong Chuan Road Fisheries Market, Shanghai Heng Da Fisheries Market, etc. from March in 2011 to December in 2012. Twenty five grams of each sample were placed in 225 ml of sterile 3.0% NaCl solution and homogenized in a stomacher (Bagmixer 400W, Interscience, St. Nom, France). 1ml of each homogenate was incubated into 9ml alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.0), diluted into seven series gradient and incubated at 37 °Cfor 16~18h with shaking. A loopful of the enriched broth was streaked onto *Vibrio* chromogenic medium (CHROMagar, Paris, France) and two purple colonies were picked and streaked onto TSA (add 3% NaCl) and incubated at 37°C overnight. Then a single colony was picked to TSB (add 3% NaCl) and incubated overnight. And the strains were preserved in duplicate by glycerol (25%) under -20°C to prepare for the further usage.

Isolation of genomic DNA

DNA was isolated using Qiagen's QIAamp DNA Mini kit in accordance with the manufacturer's instructions (Qiagen Inc., Valencia, CA, US). Isolated DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, Delaware, US), diluted to a standardized concentration (200 ng/ml) in 16T low E buffer (10 mM tris, 0.1 mM EDTA, pH 8.0) and stored at -20°C.

PCR confirmation of total and pathogenic *V. parahaemolyticus*

Three sets of oligonucleotide primers (*tlh*-F/*tlh*-R,*tdh*-F/*tdh*-R,*trh*-F/*trh*-R) (Table1) were designed to detect *V. parahaemolyticus* in possible strains and virulence genes, *tdh*, *trh* in *V. parahaemolyticus* strains and the PCR conditions were listed in (Table 2).

Determination of antibiotic susceptibility

The antimicrobial susceptibility tests were performed essentially by the disc diffusion method as described by CLSI with disc containing antibiotic (Oxoid Ltd., England). Antibiotics tested are Amoxicillin-clavulanic acid (AMC 30μg), Cefotaxime (CTX 30μg), Ceftazidime (CAZ 30μg), Amikacin (AK 30μg), Gentamicin (CN 10μg), Ciprofloxacin (CIP 5μg), Chloramphenicol (C 30μg), Tetracycline (TE 30μg), Levofloxacin (LEV 5μg), Trimethoprim-sulfamethoxazole (SXT 25μg).

ERIC-PCR

A pair of 22-mer primers: ERIC 1R (52 -ATG TAA GCT CCT GGG GAT TCA C32) and ERIC 2 (52 -AAG TAA GTG ACT GGG GTGAGC G-32) were used as previously reported (Versalovic *et al*. 1991). The reaction mixture (25 µl per reaction) consisted of 10× PCR reaction buffer, 2.5 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10µM each primer, 1.0 U of AmpliTaq, and 200 ng of template DNA. PCR was performed in Eppendorf AG 6325 using the following procedure: 1 cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 1 min, and 72 °C for 5 min. PCR was completed with a final extension at 72 °C for 10min. The PCR products were separated by electrophoresis in 1.5% agarose gels followed by ethidium bromide staining and photography with a UV transilluminator (Bio-Rad).

RESULTS AND DISCUSSION

41 *tdh* positive strains were isolated and assayed to evaluate their susceptibility to ten antibiotics (Table 3). Isolates were resistant to amikacin (14.63%), gentamicin (4.88%), ciprofloxacin (2.44%), levofloxacin (2.44%), amoxicillin-clavulanic acid (2.44%), and they were susceptible to the other tested antibiotics. Amikacin and amoxicillin-clavulanic acid are categorized into β-lactam, gentamicin into aminoglycosides and ciprofloxacin and levofloxacin into quinolones. Other research also showed that *V. parahaemolyticus* was resistant to these three kinds of antibiotics. In USA, the breadth of resistance to antibiotics was unexpectedly high with 24% isolates demonstrating resistance to 10 or more agents. A significant fraction of isolates were resistant to diverse β-lactams, aminoglycosides, and other classes of antibiotics (Baker-Austin *et al*. 2008). As for amikacin, Mexico (Molina-Aja *et al*. 2002) showed 16.7% resistant rate which was in agreement with present study. While in Korea, it was reported that amikacin resistance in 95.8% isolates were observed that was much higher than our study (Jun *et al*. 2012). The high susceptibility to ceftazidime, cefotaxime, tetracycline indicated that these first-line drugs remained highly effective against pathogenic *V. parahaemolyticus* which was also confirmed by Feifei Han (Han *et al*. 2007) and Yutaka Yano(Yutaka *et al*. 2013). While, other report showed that the resistance rate to tetracycline is rather high (Kitiyodom *et al*. 2010) which may resulted from the common usage of this antibiotic in fisheries. Obviously, antibiotic resistance rate obtained in our study is lower than the other studies who aimed at total *V. parahaemolyticus* antibiotic resistance test (Jun *et al*. 2012; Ceccarelli *et al*. 2006).To our knowledge, there is no specific study on antibiotic resistance of pathogenic *V. parahaemolyticus*, and there may exist special mechanisms about antibiotic resistance in pathogenic strains.

Table 1. Names and sequences of primers

Target gene	Primer	Sequence(5'→3')	Amplication size	Reference
tlh	TLH-L	aaa gcg gat tat gca gaa gca ctg	450bp	(Bej *et al.,* 1999)
	TLH-R	gct act ttc tag cat ttt ctc tgc		
tdh	TDH-L	gta aag gtc tct gac ttt tgg ac	269bp	(Bej *et al.*, 1999)
	TDH-R	tgg aat aga acc ttc atc ttc acc		
trh	TRH-L	ttg gct tcg ata ttt tca gta tct	500bp	(Bej *et al*., 1999)
	TRH-R	cat aac aaa cat atg ccc att tcc		

Table 2. Conditions for amplification profiles

Gene	denaturation	annealing	elongation	cycling times
tlh	94-4 min	60-1min	72- 5min	25
tdh	94- 11min	60- 1min	72- 11min	25
trh	94- 4min	60-1min	72- 5min	25

Table 3. Antibiotic susceptibilities of pathogenic *V. parahaemolyticus* in seafood in Shanghai

Antibiotic	Sensitivity (S)		Intermidiate (I)		Resistance(R)	
	No.	(%)	No.	(%)	No.	(%)
AMC	39	95.35%	1	2.33%	1	2.44%
CTX	38	90.70%	3	9.30%	0	0.00%
CAZ	40	97.67%	1	2.33%	0	0.00%
AK	25	60.47%	10	25.58	6	14.63%
CN	29	69.77%	10	25.58%	2	4.88%
CIP	35	83.72%	5	13.95%	1	2.44%
C	41	100%	0	0.00%	0	0.00%
TE	41	97.67%	1	2.33%	0	0.00%
LEV	39	95.35%	1	2.33%	1	2.44%
SXT	40	97.67%	1	2.33%	0	0.00%

Molecular typing is used for epidemiological studies as it provides the information on genetic relatedness of different bacterial strains, the source of infection, molecular markers of virulent and host specific strains (Olive *et al*. 1999). By analyzing the relationship between the amount and distribution of the fragments in bacterial genome, pathogenic strains could be classified into different categories.

As demonstrated by fig.1, we identified six patterns in similarity dice of 0.75. In our study these pathogenic strains have the high similarity though isolated from different location and different samples, which indicated that these strains may come from the same pandemic stains. Moreover, 18 strains can be classified with Vp1 that indicated much more potential risk as Vp1 was the pandemic strain. And 16 strains can be classified with Vp2. In other hand, Vp21,Vp61, Vp26 was single category which may include some special information for further research to pathogenic *V. parahaemolyticus*.

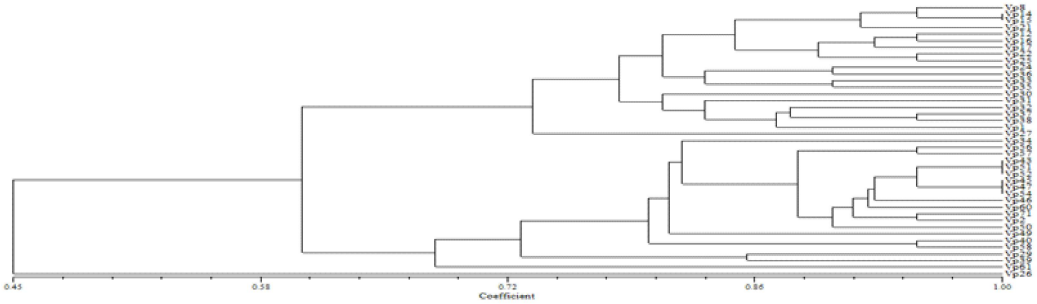

Fig 1. UPGMA dendrogram illustrated the clustering of amplification patterns of the 41 pathogenic *V. parahaemolyticus* isolates with ERIC-PCR using SLT-Ntsys-2.10e

Overall, ERIC-PCR cannot differentiate the susceptible and resistant pathogenic strains (Data not show). That is to say, clustering based on ERIC-PCR did not coincide with the isolation sources or patterns of antibiotic resistance which was similar with what was found in Korean seafood (Jun *et al*. 2012).

CONCLUSION

The pathogenic *V. paraheamolyticus* strains isolated from three kinds of seafood products in Shanghai seem not having much high antimicrobial resistance though some of them were resistant to three kinds of antibiotic. And the ERIC-PCR method cannot differentiate the susceptible and resistance pathogenic strains. This research can provide supplement data for risk assessment of *V. parahaemolyticus*.

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